SURFACE MODIFICATION FOR BIOCOMPATIBILITY

Contract No. NS 5-2322

Quarterly Progress Report #4

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The University of Michigan

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Overview

This report is a summary of our activity in the fourth quarter of 1995. In the fourth period this year we continued our efforts to deposit and characterize microstucturally controlled bioactive protein polymer films on solid surfaces and their biological activity in vivo. We have also now obtained quantitative information about the electronic properties of the coatings using Impedence Spectroscopy, in collaboration with Prof. David Anderson's group (Jim Weiland) in the EECS department. We also report success in the patterning of the proteins on solid substrates, in collaboration with Khalil Najafi in the EECS department. This report provides an overview of the major results to date and discusses our plans for the future. We have been working to evaluate (1) protein polymer film deposition and morphology, (2) bioactivity of protein polymer films *in vitro*, and (3) bioactivity and stability of protein polymer films *in vivo*. We also describe our efforts to discuss our work in (4) external communications with the scientific community.

1. Protein Film Deposition and Morphology

Progress:

Figures 1 and 2 show extended data sets for cell adhesion on substrates coated from SLPF solutions of various concentrations. The data (Figure 1) show that the total number of cells and adhered cells increases with increasing protein content on the surface. The amount of protein deposited increases with concentration, both in terms of the fraction of area coverage and the average thickness (Figure 2).

In previous work in our laboratory, we confirmed that copper grids could be used a shadow-masks to create templated regions of polymer on the solid substrate. In collaboration with Khalil Najafi, SLPF was patterned onto silicon substrates using phtoresist as a shadow mask. In this approach the photoresist is dissolved away using acetone, which is a non-solvent for the polymer. This approach significantly improves the versatility and resolution of the patterns we can produce. Figures 3 and 4 are scanning electron micrograph (SEM) images of beaded (Figure 3) and fibrous (Figure 4) morphologies that can be patterned by this approach. *In vitro* studies are underway.

In collaboration with investigators in the Kresge Hearing Research Institute, we have explored techniques for depositing proteins on silicone rubbers (Silastic) used in biomedical applications such as cochlear prostheses. While silcon wafers and probes are stiff, the silicone rubber is soft and pliable. These variations in mechanical properties place different constraints on the optimal morphology and properties of the coating. A thick, continuous coating does not appear to adhere well to the silicone substrate (Figure 5).

Thinner, more pliable coatings should be more appropriate for this material (Figure 6). *In vitro* studies are underway.

Our ability to characterize the electrical performance of the probes continues to develop. Figures 7 and 8 show the amplitude of the impedence across a single site (site 2 on electrode sx04). The data show the systematic decrease in impedence with increasing frequency, as found in other organic thin films. After activation, there is a significant decrease in the impedence at frequencies below ~1000 Hz, and an increase in the impedence above ~1000 Hz. The rate at which the impedence changes with increasing frequency appears to be different for the activated probes. Coating the activated probes appears to increase the impedence, particularly at intermediate frequences.

Further studies are underway to confirm the reliability and reproducibility of these results. In particular, we are depositing films with various thicknesses to establish the relationship between film structure and performance. Furthermore, we are examining the morphology of the substrates before and after depsositon by electron microscopy.

Plans:

We are continuing our systematic studies of probe electrical performance as a function of coating thickness and morphology. We are also continuing detailed studies of protein film morphology by low voltage scanning electron microscopy, atomic force microscopy, and transmission electron microscopy. We are initiating a study of the microstructure of the probe by TEM, particularly near the iridium site before and after activation.

2. Bioactivity of Protein Polymer Films *in vitro*

Progress:

Efforts to evaluate the biological response of the protein films continue. In particular, we are actively investigating the ability of Schwann cells to selectively adhere to patterned substrates. These studies will need to consider the possible role of any residual photoresist or solvent on cells *in vitro*.

Studies of Schwann cell adherence to SLPF coated silastic substrates confirm that the performance of these coatings are not yet optimized. Schwann cells were grown in vitro in roller bottles in L15 medium for 18 h, then visualized using toluidine blue, anti-lial fibrillary protein antibody, or using the chromosomal dye propdium iodide. Only rarely was it possible to find patches of adherent cells.

3. Bioactivity of Protein Polymer Films *in vivo*

Progress:

As we discussed in our last report, polypropylene suture (~50 micron diameter) was coated with the following materials and implanted in the Guinea Pig CNS:

1. no coating (control)
2. SLPF coated
3. SLPL coated

4. SLPF/Schwann cells
5. SLPF coated and exposed to CSF
6. SELP coated

Our earlier optical microscopy studies are now beginning to be complimented by TEM studies of three-week embedded polymer coated suture.

Plans:

The in vivo studies are ongoing, and nine-month embedded implants will be available for examination in the near future. We are investigating the ability of the Edge microscope (which allows for significantly better depth of focus than conventional scopes) to examine astrocyte behavior near an implanted probe.

4. External Communications

An preprint titled "Electric Field Mediated Deposition of Bioactive Polypeptides on Neural Prosthetic Devices", by Chris Buchko, Ken Kozloff, Atisa Sioshansi, K. Sue O'Shea, and David C. Martin was submitted for a proceedings volume for the fall 1995 Materials Research Society meeting in Boston, MA. Chris Buchko presented the paper at the meeting. A copy of this paper is enclosed.

The invited review paper on *Processing and Characterization of Silk-like Protein Polymers*, by David C. Martin, J. Philip Anderson, Chris Buchko, and Tao Jiang, which is scheduled to appear in a special volume on *Protein Polymer Materials*, edited by Kevin McGrath and David Kaplan of the U. S. Army Natick RD&E Center, is essentially complete and ready for submission.

Fig 1

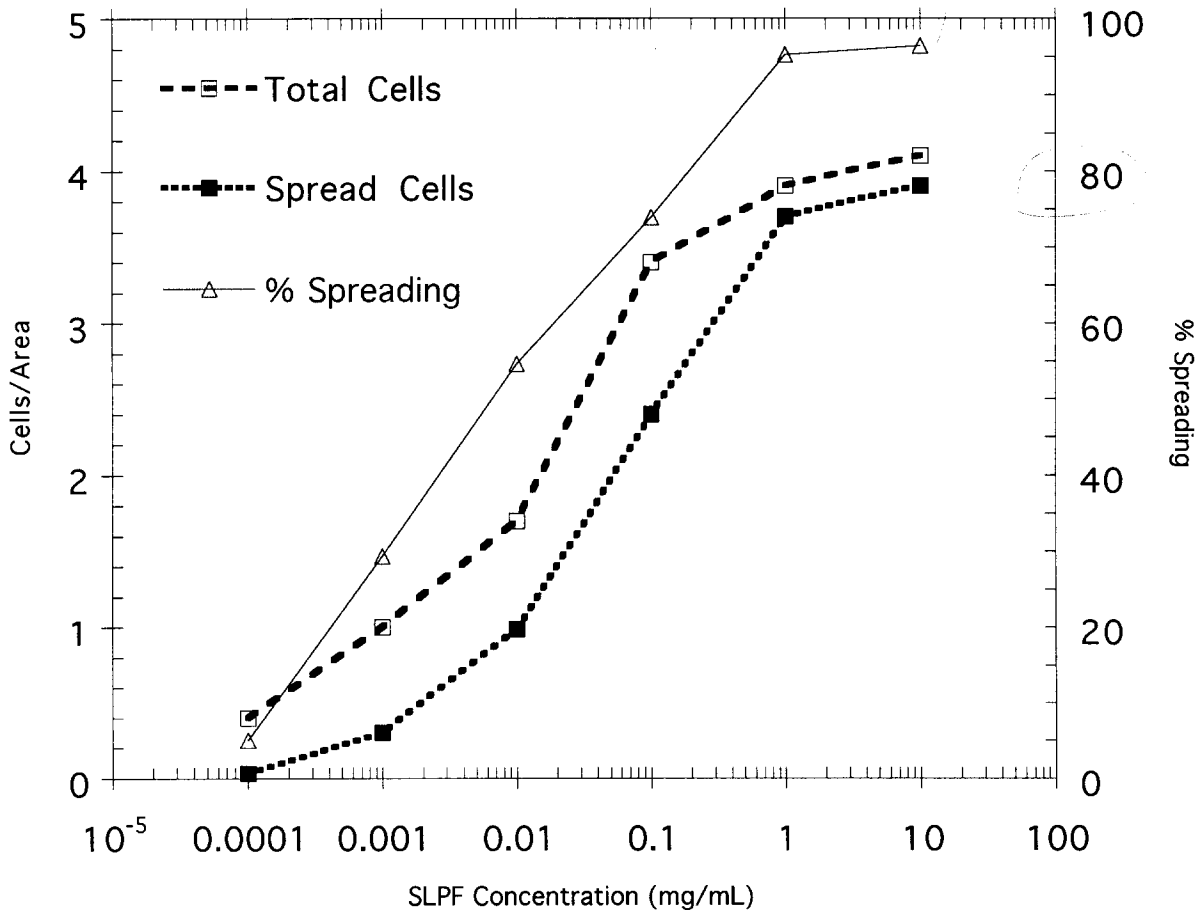
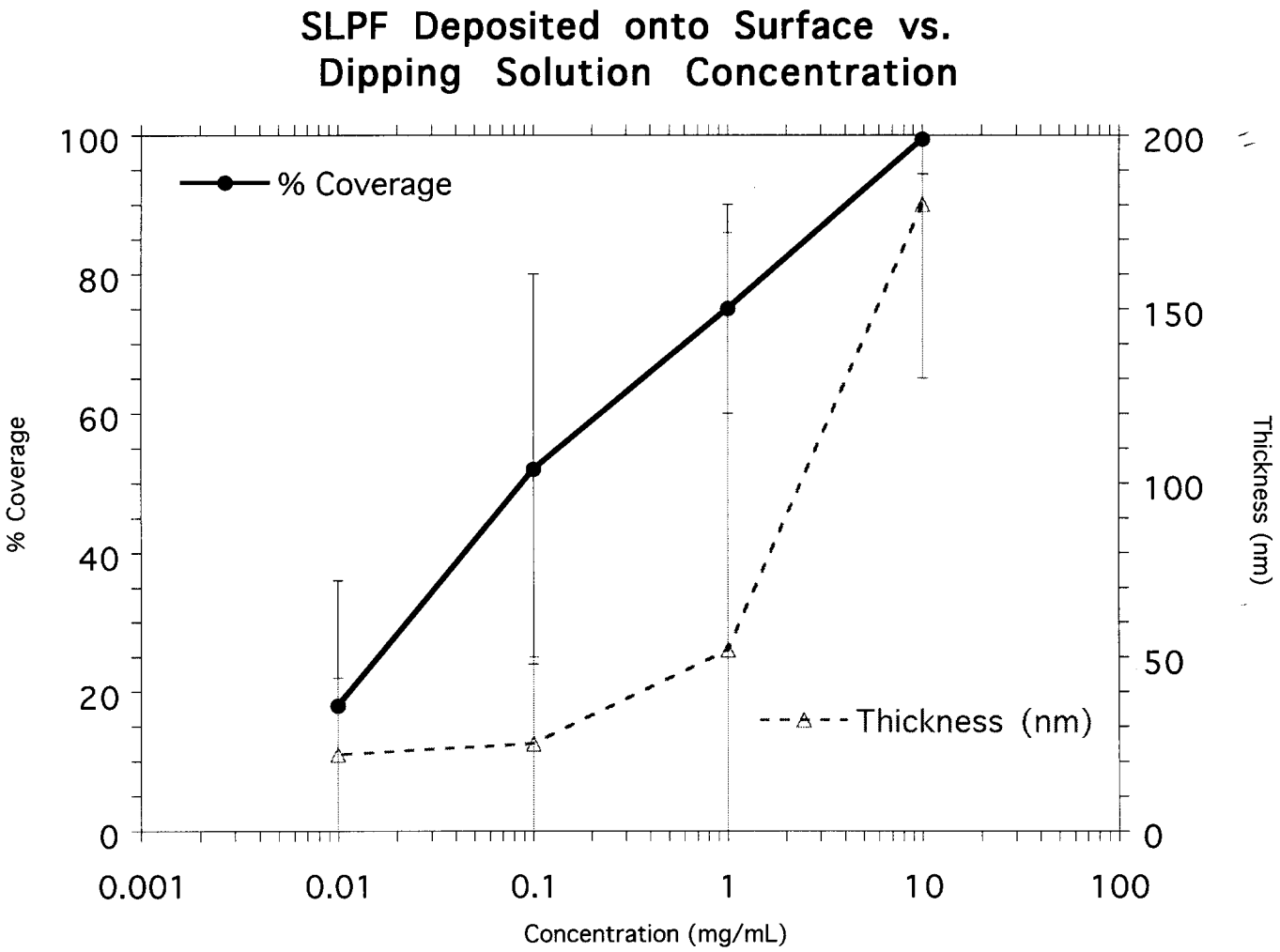

Cell Adhesion vs. Dipping Solution Concentration
Total Cells
Spread Cells
% Spreading
Cells/Area
% Spreading
SLPF Concentration (mg/mL)
0
1
2
3
4
5
0
20
40
60
80
100
10-5
0.0001
0.001
0.01
0.1
1
10
100

FIG 1

Fig 2


SLPF Deposited onto Surface vs.
Dipping Solution Concentration
% Coverage
Thickness (nm)
100
80
60
40
20
0
200
150
100
50
0
0.001
0.01
0.1
1
10
100
Concentration (mg/mL)

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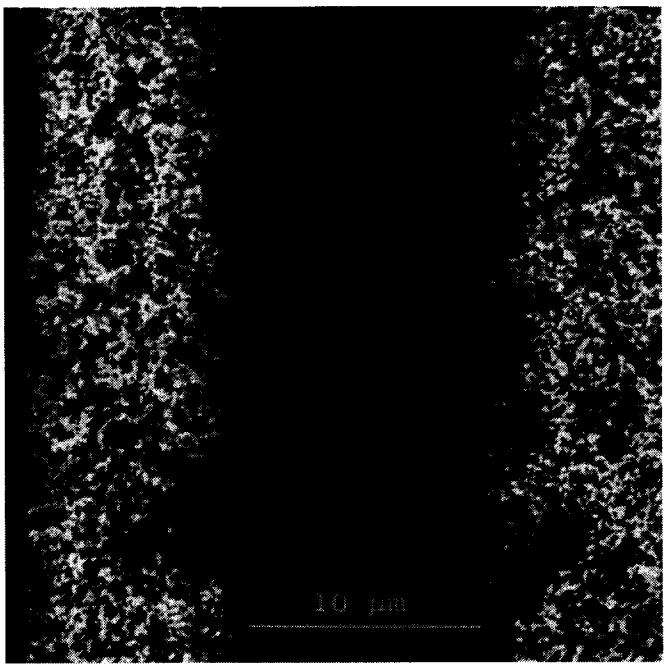
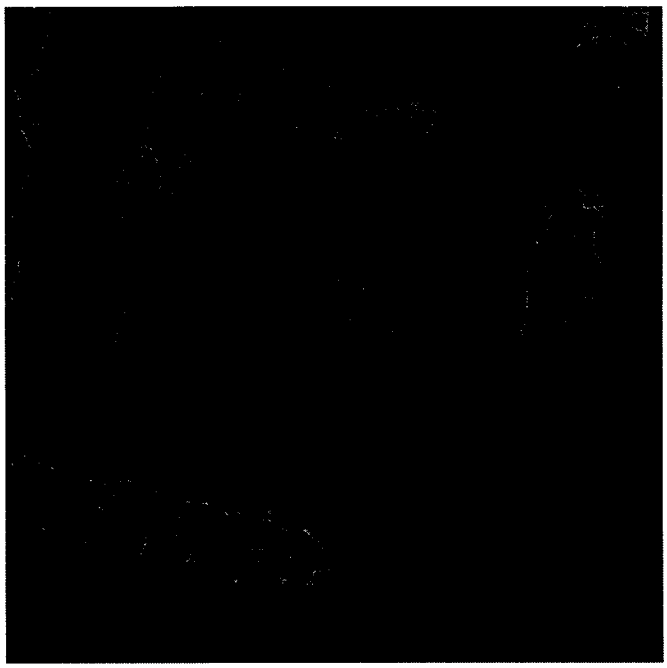


Patterned SLPF droplets on silicon substrate

FIG 3

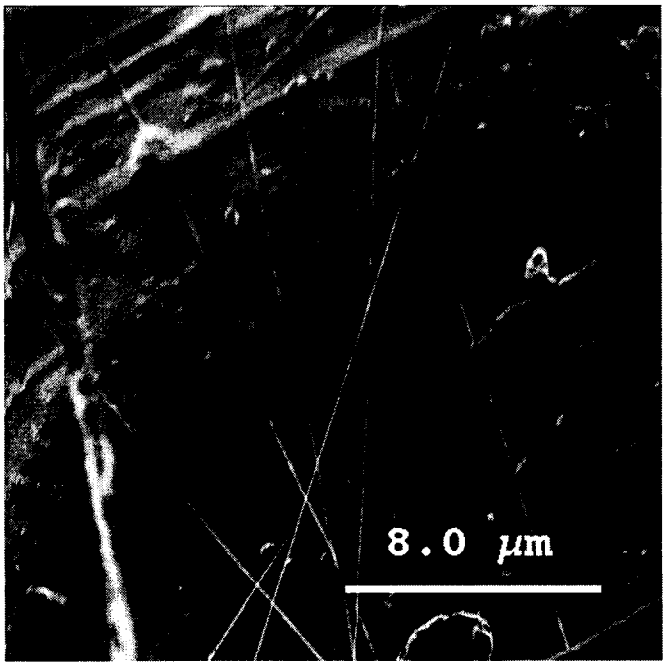


Patterned SCPF filaments on silicon substrate

FIG 4

SLPF coating on silicone rubber substrate

Fig 5



SLPF filaments on silicone rubber substrate

FIG 6

Probe Impedance

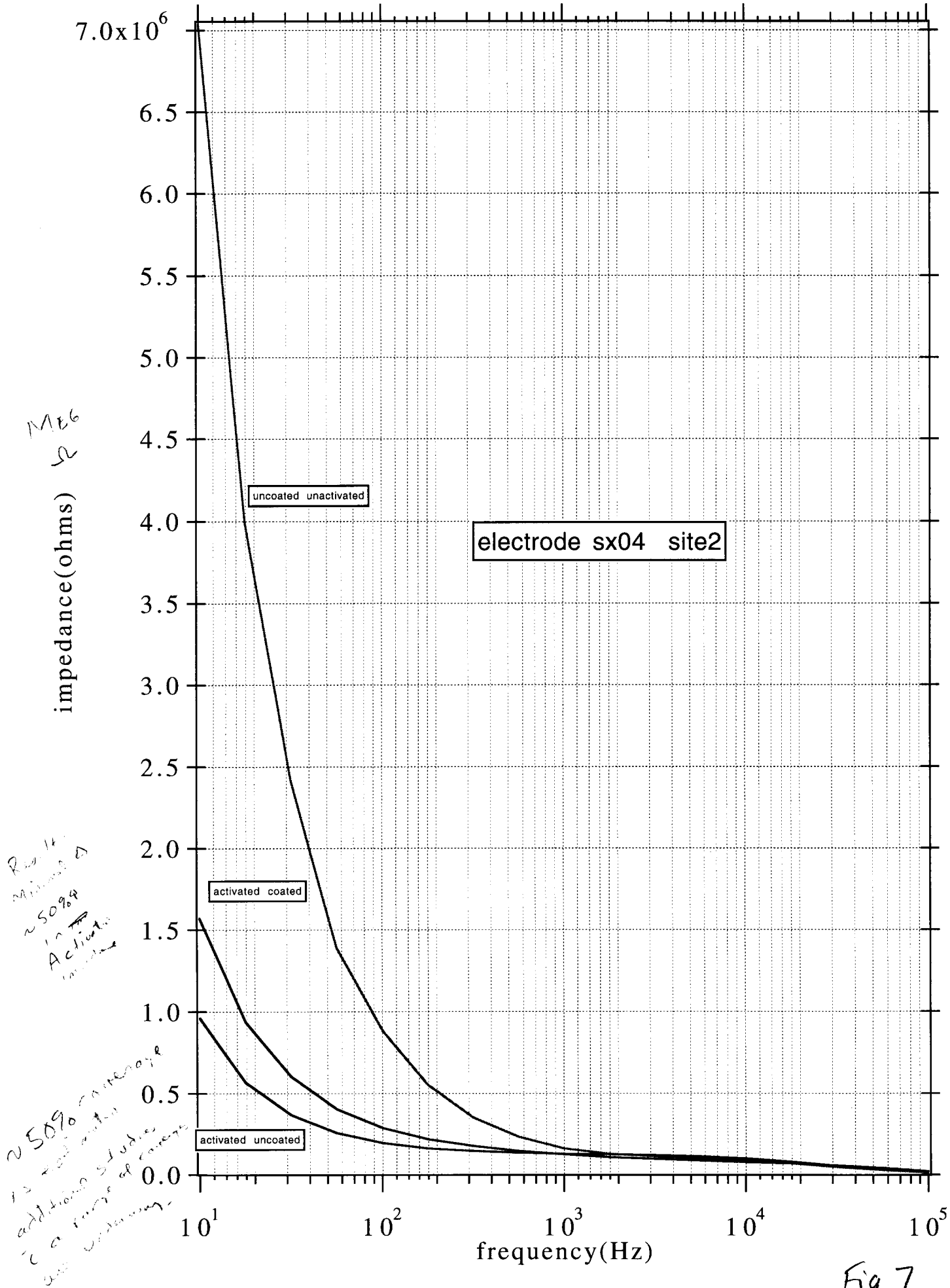


Fig. 7

Probe Impedance

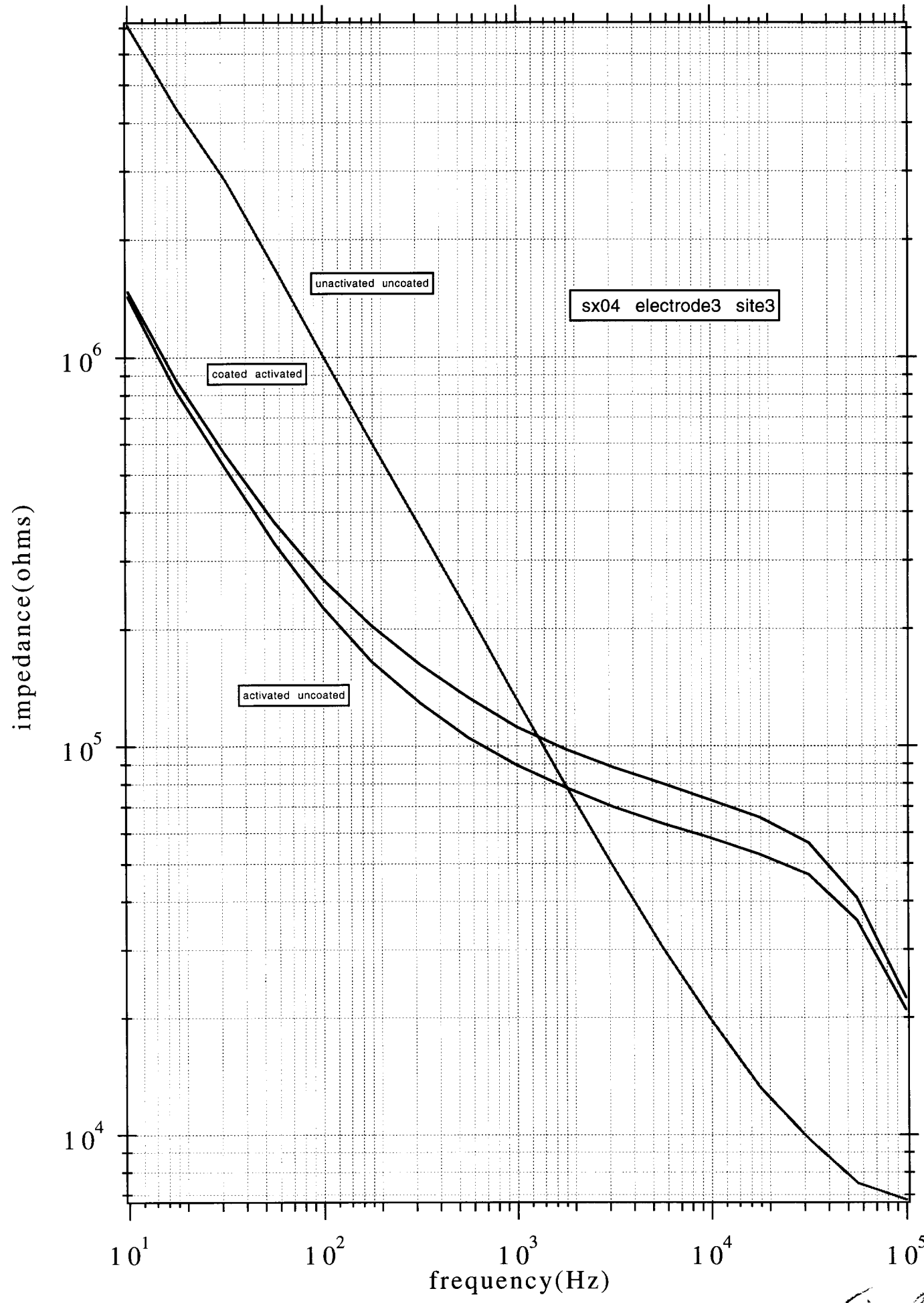


Fig 8

ELECTRIC FIELD MEDIATED DEPOSITION OF BIOACTIVE POLYPEPTIDES ON NEURAL PROSTHETIC DEVICES

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ABSTRACT

We have developed processing schemes for depositing three-dimensionally tailored layers of protein polymers on a variety of solid substrates. One of our goals is to create stable, biocompatible coatings on silicon devices for implantation in the central nervous system. Our research has identified several candidate coatings whose morphologies lie in the biologically significant 0.1 to 100 micrometer length scale. Using electric field mediated deposition, we are able to process polypeptides into biologically-responsive films and coatings. Quantitative analysis of the structural evolution of the coating enables us to fine-tune its morphology by varying the field strength and geometry or solution concentration. The interaction of the coated substrates with neurons and glial cells are examined *in vivo* and *in vitro*. Data collected from light optical microscopy, atomic force microscopy, transmission electron microscopy, and scanning electron microscopy provide insight about the relationship between the microstructure of these coatings and their macroscopic properties.

INTRODUCTION

Materials scientists are constantly manipulating the microstructure of materials in order to tune physical properties toward a specific application. The advent of genetic engineering allows molecular engineers to design precisely-defined polypeptides with combinations of properties previously unknown in nature. The next step in the development of these materials is to devise appropriate processing schemes to optimize the desired properties. The polypeptides used in this study are genetically engineered to contain not only selected cell binding domains from naturally occurring extracellular matrix proteins such as fibronectin and laminin, but also silk-like sequences to provide structural integrity. The biofunctional portion of these molecules requires accessibility to the target cells and the structural segments promote the formation of thermally and mechanically stable oriented crystalline domains. An electric field mediated processing technique, electrostatic fiber formation, produces fibrous networks of solid polypeptide fibers with diameters as small as 50 nanometers. These tiny filaments show microstructural orientation and exhibit a large surface-area-to-volume ratio.

Three-Dimensionally Tailored Films and Coatings

The fibrous, nonwoven networks resulting from electrostatic fiber formation are an example of structures that can be described as three-dimensionally tailored. In general, a three-dimensionally tailored film or coating is one that exhibits a gradient in properties throughout its thickness over a length scale that is relevant to its application. In this instance, the biologically relevant length scale is 0.1 to 100 micrometers. Included among the properties of interest in biological applications are porosity, mechanical strength, biofunctionality, biodegradability, and timed drug release. Gradients in porosity and mechanical strength can be produced by controlling the density of coating elements, such as fibers, in successive layers of the structure. Varying the porosity allows for control over the extent to which cells become incorporated in the coating, as well as governing transport through the coating. The tremendous difference in modulus between soft, living tissue (modulus ~10 MPa) and a stiff implanted device (modulus ~200 GPa) can perhaps be accommodated by a gradient in mechanical properties. Kinetically controlled drug

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release can be governed by the diameters of the fibers in which the compound is suspended, and also by the porosity of the coating.

Properties such as biofunctionality and biodegradability can be changed by processing as well as by materials synthesis. Genetic engineering can produce known combinations of biofunctionality in a given molecule to achieve the desired proportion of each property. Alternately, multilayer coatings of different polypeptides can be produced using electric fields.

Three-dimensionally tailored films and coatings of protein polymers serve as scaffolds that stimulate and support cellular growth, with the possibility of programmed degradation once the implant site becomes stable. In the case of biocompatibilizing implanted devices, it can be highly advantageous not just to prevent catastrophic tissue response with a biologically inert coating, but to provide an active framework for biological interaction with the device. An intimate union between the coating and the surrounding tissue could help prevent unintended physical displacement of the device in a chronic implant situation.

The emerging field of tissue engineering depends upon the fabrication of three-dimensionally tailored structures. Organs are complex assemblies of tissues with specific, spatially-organized functionality. Tissue engineering is predicated on the ability to replicate this organization by providing a scaffolding for cell culture outside the body[1]. When the tissue has reached a sufficient level of maturity, it can be implanted in the body as a functional organ, slowly making the transition from a hybrid structure to complete tissue as more cells grow and the scaffold degrades in a controlled way. It is essential to be able to design self-supporting structures onto which target cells will reliably and specifically assemble.

Protein Polymers

Synthetic protein polymers are generated by recombinantly modified *E. Coli.* This technology enables the construction of polypeptides with precisely defined amino acid sequences. Sets of sequences make up modular subunits that exhibit technologically useful properties. A silk-like amino acid sequence can provide structural stability while and a sequence from naturally-occurring fibronectin can stimulate cellular adhesion. This combination is found in the primary material used in this study, SLPF (ProNectin® F) [2]. Additionally, functional modular units providing the flexibility of elastin or the cell-specific binding sites of proteins like laminin have been incorporated in this family of silk-like protein polymers.

Much of the work in our group has focused on the microstructure of SLPF and SELP (silk and elastin containing polypeptides) [3]. It is evident from this work that the fibronectin binding sites are excluded from the crystalline silk domains, efficiently forming a functionalized surface. The hydrophobic nature of these proteins promotes a strong adhesion to glass and silicon substrates, and the structural stability of the silk backbone allows for processed protein films to be autoclaved without losing biological activity. The molecular weight of SLPF is 73 kD.

Electric Field Mediated Deposition

Electrostatic atomization and electrostatic poling are two types of electric field mediated deposition. Electrostatic atomization occurs when an applied electric field exerts enough force on a liquid surface to overcome the surface tension of the liquid and produce a fine spray of droplets [4]. Electrostatic fiber formation, or electrospinning, is a special case of electrostatic atomization in which the high viscosity of the polymer solution produces liquid filaments that solidify into submicron to micron sized fibers [5]. Electrostatic poling involves placing a polymer solution between charged plates and varying the applied field as the polymer solidifies, sometimes in the presence of a coagulant. This paper will address our use of electrospinning to produce polymer thin films and coatings.

The apparatus used in the electrospinning process consists of a syringe barrel, which can be pressurized, an embedded electrode wire, a 0.15 mm internal diameter syringe needle, and a substrate held in contact with a grounded wire (Figure 1). The embedded wire is held at a potential to impart a surface charge to a pendant drop of solution at the tip of the syringe needle. Depending on the viscosity of the solution and the interfacial energy between the solution and the capillary, backpressure may be required to develop the pendant drop and continuously refresh it during the fiber formation process. At a certain critical voltage, the pendant drop becomes unstable, taking on

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a conical shape. It is from the apex of this cone that discrete amounts of solution ranging from a fine mist of droplets to a stable filament are ejected.
The potential modes of pendant droplet deformation depend on bulk solution properties such as conductivity and viscosity, interfacial energies among the capillary tube, solution, and surrounding medium, and the strength and geometry of the electric field [4]. For the cone-jet mode of pendant droplet deformation, the critical voltage can be predicted by

$$V_c^2 = 4\frac{H^2}{L^2}\left(\ln\frac{2L}{R} - 1.5\right)(0.117\pi R\gamma) \qquad (1)$$

where H is the distance between the electrodes, L is the length of the capillary, R is the capillary radius, and γ is the surface tension [5].

The droplets and filaments that are ejected by the atomization process undergo further deformation due to a combination of the electrical stresses on the droplet surface and the hydrodynamic drag forces between the droplet and the surrounding medium. Droplets elongate parallel to the field direction, which is also the direction of the drag force. They can break up further into smaller droplets that are in turn deformed by the same forces. The evaporation of the solvent or the cooling of the polymer melt conspires with the deformation process to create submicron diameter fibers. Alternatively, a pendant drop that deforms into a stable filament of tens of microns will splay into successively smaller filaments until the resulting tiny filaments solidify into polymer fibers. Fibers spun from polymer melts have been collected on a an uptake spool in the manner of conventional dry-spinning [5]. It is possible to create self-supporting nonwoven meshes from polymer melts and solution, as well as lacy, porous coatings on a variety of substrates [6]. Certain extended chain polymer systems have shown a high degree of fiber orientation when electrospun [5,6].

One advantage of using electrostatic deposition for producing thin films and coatings which is particularly important in the present instance is that very little product is wasted during this process. In many coating applications, such as spin coating, dip coating, or pressurized atomization, significant mass losses occur during the process. Electrostatic deposition provides a high degree of control over polymer placement on the substrate, as well as control over the morphology of the coating. Varying the strength and geometry of the applied field also changes the coating microstructure.

Neural Prosthetic Devices

The Center for Neural Communications Technology at the University of Michigan fabricates solid state electronic devices for use in neural prostheses [7]. These devices are composed of a boron-doped silicon base, polysilicon raceways and iridium electrodes, with a multilayer silicon nitride/silicon oxide dielectric as an insulator. For chronic implantation, the coatings for these devices have a number of performance requirements. The coating must at the very least be biologically inert, but more preferably it will actively encourage tissue acceptance of the device. It must also facilitate neurite outgrowth to the stimulating/recording sites to ensure good electron transport across the tissue/device interface. In past trials, one mode of implant failure is caused by the migration of the device through tissue and away from its intended neural target. Finally, the coating must introduce a minimal impedance increase to the tissue/device interface. We believe the coating that holds the most promise for satisfying these requirements is a porous, discontinuous, and filamentous structure.

EXPERIMENT

The first step in generating these three-dimensional films and coatings is to determine the extent of structural control afforded by electric field mediated deposition. The bulk solution parameter most easily controlled is the polymer concentration in solution. While SLPF is soluble in a number of solvents, we chose formic acid because of its relatively high volatility. Changing the concentration of the solution also changes the viscosity and the evaporation rate of the solution, two important variables in the electrostatic fiber formation process. Solution concentrations in this

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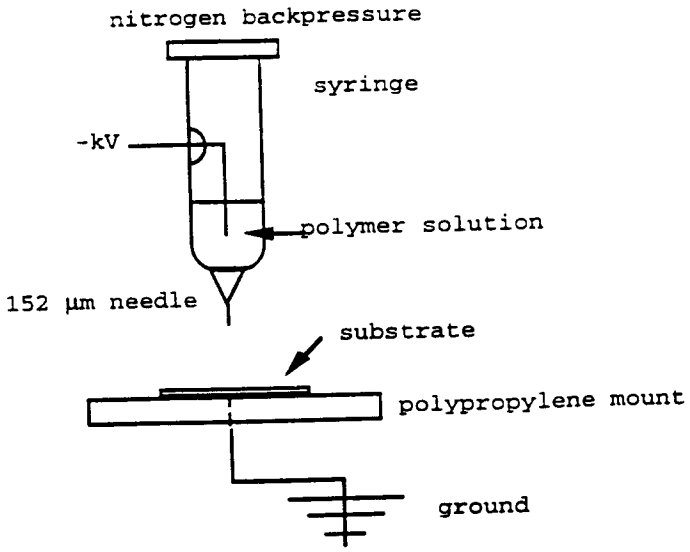

Figure 1. Schematic of the electrostatic deposition apparatus. Typical distance between needle and substrate is 20 mm.

study ranged from 100 mg/ml (~7 % by weight) to 10 mg/ml. Also, by changing the separation between the syringe needle and the substrate and varying the applied voltage, it is possible to explore the effect of the strength and geometry of the field on the process. Typical successful field strengths were between 3 kV/cm and 10 kV/cm. The resulting coatings were studied using a variety of techniques, including optical microscopy (Nikon Optiphot POL-2), scanning electron microscopy (SEM) (Hitachi S-800), atomic force microscopy (AFM) (Digital Instruments Nanoscope III Multimode Head), and transmission electron microscopy (TEM) (JEOL 2000 FX). Both the SEM and the TEM samples were coated with gold or gold/palladium to improve contrast.

Biological functionality of the coatings was tested *in vitro* and *in vivo*. The first cell adhesion experiments were carried out using a coating developed by simply dipping a coverslip into a solution of known concentration for 1 minute, and then removing the coverslip and allowing it to dry. This method was chosen for its simplicity and to provide a reference for studying the effects of coating morphology on cell adhesion. The substrates were then UV-light sterilized, cultured with Schwann cells for 2.5 hours, and fixed. Cells were counted by optical microscopy and classified as either specifically adherent or non-specifically adherent. Schwann cells and neurons were also cultured with filamentous coatings of SLPF and SLPL on silicon and glass substrates. For the ongoing *in vivo* experiments, suture is being used as a model for the implant because of its ease in sectioning for histological analysis. Pieces of suture are cut and placed over a silicon substrate during the electrodeposition process, becoming coated with protein polymer filaments (Figure 2). These pieces of suture undergo different treatments before implantation for various time periods.

RESULTS AND DISCUSSION

Structural Control

The morphology of the electrodeposited films and coatings depends on both the concentration of the polymer solution and on the strength of the electric field. At 100 mg/ml SLPF in formic acid, the coatings consist entirely of filaments (Figures 2 & 3). The texture on the filaments in both of these images indicates a degree of orientation along the fiber axis. Below 50 mg/ml, the coating consists of both filaments and spheres of solid polymer. Figure 4 shows an AFM image of a coating, processed from 20 mg/ml SLPF in formic acid, composed entirely of solid polymer particles. Since the decrease in concentration corresponds to a decrease in viscosity, the change in morphology can perhaps be accounted for by the propensity for the more viscous solution to elongate under the electrostatic and hydrodynamic stresses. The less viscous solution acts more like an atomized liquid, becoming a fine spray of droplets that solidifies as it dries. The

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wide range of sizes in the coatings can be attributed to the splaying of the electrified polymer jet. Since the liquid filaments ejected from the syringe carry the same charge, they break up irregularly during the electrodeposition process.

Using a portable mount for optical microscope objective lenses, it is possible to observe the onset of pendant droplet deformation and therefore the critical voltage required for electrospinning. At 100 mg/ml, the observed critical voltage is 4.2 kV, which is reasonably consistent with the predicted value of 3.1 kV.

As the applied field increases from 4 kV/cm to 6 kV/cm, the deposition rate changes from 0.13 μm/sec to 0.17 μm/sec. The increased field strength is analogous to increasing the draw rate in a dry-spinning apparatus. Although the large variation in fiber diameter makes measuring the average diameter difficult, it is expected that the average fiber diameter will decrease with applied field, making the analogy of polymer electrospinning to polymer dry-spinning even more appropriate.

More evidence of our ability to selectively deposit protein polymer on surfaces can be found in Figure 5. This pattern was created using a shadow-masking technique in which a thin mask was placed above the silicon substrate and then removed after the deposition process. Shadow masking is just one way to pattern the coating on a substrate. Transverse electric fields also allow for control over the charged particles and fibers that make up the coating.

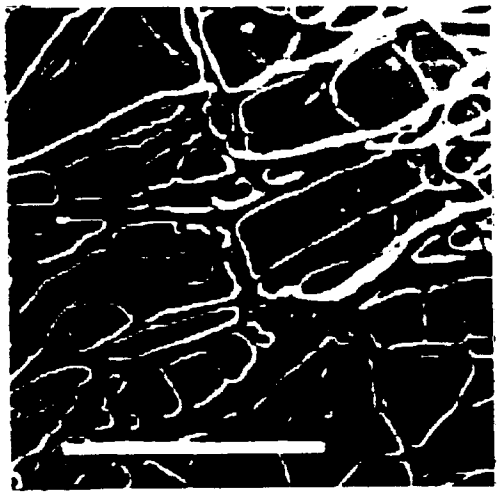

Figure 2. SEM micrograph of electrospun SLPF filaments on polypropylene suture. The scale bar is 10 μm.

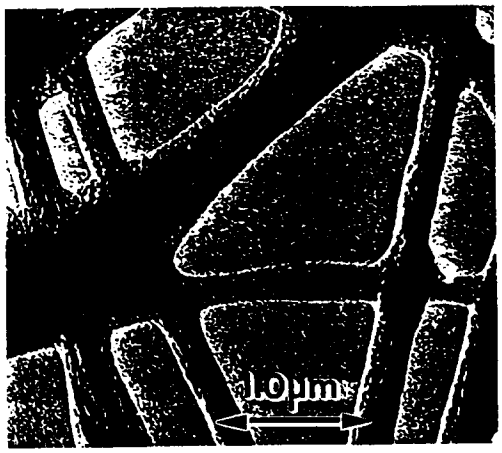

Figure 3. TEM micrograph of electrospun SLPF filaments. The scale bar is 1.0 μm

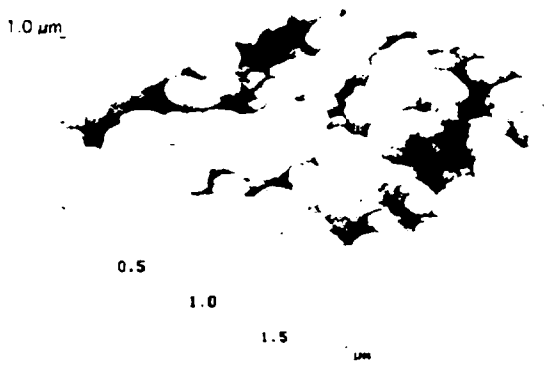

Figure 4. AFM image of electrodeposited SLPF particles on a silicon surface. The image is 2 μm X 2μm.

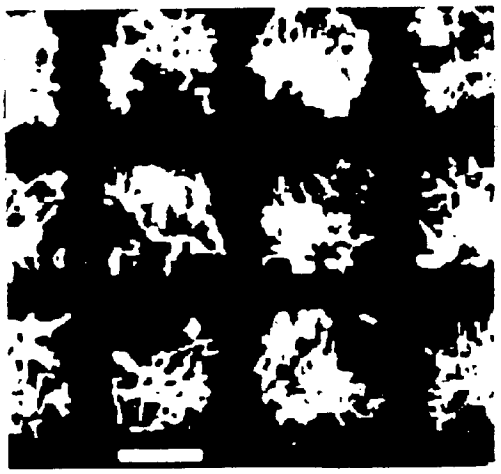

Figure 5. Dark field OM of shadow masked SLPF filamements on a silicon surface. Each square is 95 μm on a side.

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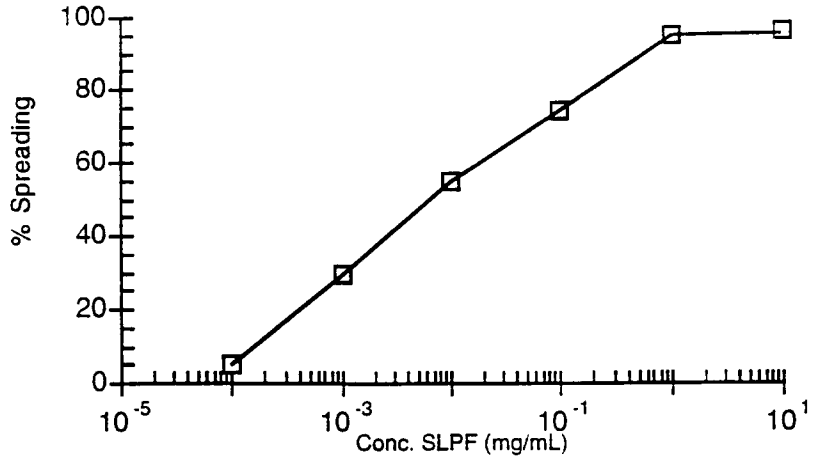

Figure 6. Percentage of cells specifically adhering to a coverslip dipped in SLPF/formic acid solution at the given concentrations and dried.

Biological Functionality

The results of the cell adhesion experiments show that dipping a coverslip into a solution whose concentration is as low as 1.0 mg/ml still provides a high degree (95 %) of specific cell adhesion (Figure 6). The thin film on the surface of the substrate as a result of the dipping covers about 75 % of the surface at an average thickness of approximately 60 nanometers. During the initial stages of cell adhesion, cells tend to extend along the length of the filaments before reaching their final, fully spread state. Also, neurons showed good specific adhesion to SLPL.

CONCLUSIONS AND FUTURE WORK

By varying the concentration of protein polymer in solution and the strength of the electric field, it is possible to manipulate the morphology of the deposited coating using electrostatic fiber formation techniques. This process provides control over patterning and the placement of the polymer and minimizes material consumption. There is no adverse effect on the biological activity during electric field mediated deposition It is anticipated that the high surface-area-to-volume ratio of the filamentous coating will prove a more efficient cellular adhesive than thin films. Our *in vivo* results to date show a favorable neural tissue response, and the incorporation of small molecules into the coatings has proved successful.

ACKNOWLEDGEMENTS

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